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Do bacterial cell numbers follow a theoretical Poisson distribution? Comparison of experimentally obtained numbers of single cells with random number generation via computer simulation

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ABSTRACT

We investigated a bacterial sample preparation procedure for single-cell studies. In the present study, we examined whether single bacterial cells obtained via 10-fold dilution followed a theoretical Poisson distribution. Four serotypes of *Salmonella enterica*, three serotypes of enterohaemorrhagic *Escherichia coli* and one serotype of *Listeria monocytogenes* were used as sample bacteria. An inoculum of each serotype was prepared via a 10-fold dilution series to obtain bacterial cell counts with mean values of one or two. To determine whether the experimentally obtained bacterial cell counts follow a theoretical Poisson distribution, a likelihood ratio test between the experimentally obtained cell counts and Poisson distribution which parameter estimated by maximum likelihood estimation (MLE) was conducted. The bacterial cell counts of each serotype sufficiently followed a Poisson distribution. Furthermore, to examine the validity of the parameters of Poisson distribution from experimentally obtained bacterial cell counts, we compared these with the parameters of a Poisson distribution that were estimated using random number generation via computer simulation. The Poisson distribution parameters experimentally obtained from bacterial cell counts were within the range of the parameters estimated using a computer simulation. These results demonstrate that the bacterial cell counts of each serotype obtained via 10-fold dilution followed a Poisson distribution. The fact that the frequency of bacterial cell counts follows a Poisson distribution at low number would be applied to some single-cell studies with a few bacterial cells. In particular, the procedure presented in this study enables us to develop an inactivation model at the single-cell level that can estimate the variability of survival bacterial numbers during the bacterial death process.

1. Introduction

Predictive modelling that takes into account the individual heterogeneity and uncertainty of bacterial behaviour has recently received increasing attention. A definition of variability and uncertainty is described as follows (Begg et al., 2014). Variability is quantified by a distribution of frequencies of multiple instances of the quantity, derived from observed data. Examples of source of variability are individual cell heterogeneity, which cause different responses among cells in a same condition and number of bacterial cells caused by naturally occurring randomness. In contrast, uncertainty refers to the unknown, single, true value of some quantity. Uncertainty arises from multiple sources, such as statistical variation, approximation, subjectivity in measurement techniques, disagreement, variability and practical unpredictability. A deterministic approach results in limited predictions of bacterial behaviour at low bacterial numbers because this approach does not consider uncertainty and individual heterogeneity (Koutsoumanis and Lianou, 2013; Membré et al., 2006). The phenomenon of tailing described in bacterial death/inactivation kinetics has not yet been predicted well, which may represent the uncertainty and variability of surviving bacterial behaviour. It has been reported that in small populations, such as less than 100 cells, individual cell behaviours become distinct from population behaviour (Aspridou and Koutsoumanis, 2015). During bacterial inactivation or growth process, bacterial behavior would be difficult to predict by point

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estimation due to the variability and uncertainty. To estimate bacterial numbers in foods contaminated at low levels, the variability of bacterial behaviour should be combined into a predictive model. A probabilistic approach is indispensable for appropriately describing the uncertainty and variability of bacterial behaviour.

Salmonella enterica and enterohaemorrhagic *Escherichia coli* can cause foodborne illness following the ingestion of less than 100 cells (Hara-Kudo and Takatori, 2011). Thus, it is important to appropriately estimate the behaviour (growth and/or death) of small bacterial numbers, such as less than 100 cells, for risk assessment of foodborne illness. Because of variability and uncertainty of bacterial behavior, it is difficult to assess the frequency of bacterial survival in foods contaminated at low levels.

Working at the single-cell level requires the preparation of single bacterial cells. However, obtaining single cells is difficult due to the statistical uncertainty associated with current experimental techniques (Hedges, 2002). In a previous study, a protocol for isolating single cells using two-fold dilutions was developed (Francois et al., 2003), and the individual lag phase of *Listeria monocytogenes* was evaluated (Francois et al., 2005, 2006). In contrast, there has been only one report on bacterial inactivation models at the single-cell level (Aspridou and Koutsoumanis, 2015). One of the reasons for the limited number of such studies is the difficulty involved in obtaining exactly one cell with high reproducibility. For single-cell-level bacterial inactivation studies, single bacterial cell samples must be stably and reproducibly prepared, and the probabilistic distribution of the cell numbers should be known. Obtaining stable single-cell populations following a mathematically described distribution would enable us to simulate the variability of bacterial cell numbers.

The Poisson distribution has been considered to provide a good fit for bacterial cell number when obtained under carefully controlled experimental conditions (El-Shaarawi et al., 1981). It is assumed that a bacterial cell number following Poisson distribution will be experimentally obtained via a dilution series. To evaluate the individual lag phase, a single-cell-level study of *Listeria monocytogenes* was performed previously; the study dealt with a few bacterial cell populations that followed a Poisson distribution (McKellar and Knight, 2000; Robinson et al., 2001). However, the bacterial cell numbers in the prepared inocula were not directly counted in these experiments, and the accuracy with which bacterial cell numbers followed the Poisson distribution remained unclear. Other studies conducted with low-level bacterial populations have not experimentally demonstrated whether bacterial cell counts follow a Poisson distribution. If a distribution of bacterial cell numbers is mathematically described, variability of bacterial cell numbers would be available.

A Poisson distribution applies to various phenomena with discrete properties whenever the probability of the phenomenon is constant in time or space. Experimental data have been estimated to fit a Poisson distribution in other fields of study, such as the distribution of seismic hazards (Wang and Chang, 2015). Thus, a Poisson distribution would fit to a random distribution in nature.

The objective of the present study was to develop a protocol to obtain single cells following Poisson distribution for use in various single-cell studies. We investigated whether single bacterial cells obtained via 10-fold dilution followed a theoretical Poisson distribution. An inoculum of each pathogenic microorganism was prepared in 10-fold dilution series to obtain bacterial cell counts with mean values of one or two. Furthermore, to examine the validity of the parameters of the Poisson distributions obtained from bacterial cell counts, we compared these parameters with those of a Poisson distribution, having used random number generation in a computer simulation. The fact that bacterial number distributions follow a Poisson distribution at low number would be applied to some single-cell studies. In particular, the procedure presented in this study would enable us to develop an inactivation model that simulates the variability of survival bacterial numbers in a stochastic inactivation process.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains were kindly provided by the Research Institute for Microbial Diseases (RIMD) of Osaka University, the Hokkaido Institute of Public Health (HIPH), and the Aomori Prefectural Research Laboratory of Public Health. Three strains of *Escherichia coli* O111 (RIMD 05092013, RIMD 05092017, and RIMD 05092026), two strains of *E. coli* O26:H11 (RIMD 05091996 and RIMD 05091997), four strains of *E. coli* O157:H7 (RIMD 0509939, RIMD 05091896, RIMD 05091897, and HIPH 12361), a strain of *Salmonella* Stanley (RIMD, 1981001), two strains of *S.* Typhimurium (RIMD, 1985007 and RIMD, 1985009), a strain of *S.* Chester (from Aomori), a strain of *S.* Oranienburg (from Aomori), and six strains of *Listeria monocytogenes* (ATCC, 19111, ATCC, 19117, ATCC19118, ATCC 13932, ATCC 15313, and ATCC 35152) were used. All strains were maintained at −80 °C in brain heart infusion broth (Merck, Darmstadt, Germany) containing 10% glycerol. A sterile metal loop was used to transfer the frozen bacterial cultures by scratching the surface of the frozen culture into 5 ml of tryptic soy broth (Merck, Darmstadt, Germany) in a sterile plastic tube. The cultures were incubated without agitation at 35 °C for 24 h and transferred using a loop inoculum at two successive 24-h intervals to obtain a more homogeneous and stable cell population. Grown cells were collected via centrifugation (1000 × *g*, 10 min at 25 °C), and the resulting pellet was washed with 0.1% peptone water twice and subsequently resuspended in 5 ml of 0.1% peptone water. To generate a single sample of each pathogen comprising every strain, equal volumes of the cell suspensions from multiple strains of each pathogen were combined to achieve an approximately equal population of each strain.

2.2. Preparation of single cells

Single cells of each pathogenic bacterium were prepared via 10-fold dilutions of bacterial cultures. The initial cell count was assumed to be approximately 10^9 CFU/ml. The inoculum was further diluted (10-fold dilution series) to obtain a 10^3 CFU/ml solution. An aliquot of 10^4 CFU/ml solution was stored at 5 °C for 24 h. The bacterial cell number was determined via direct plating of 100 μl of inoculum of a 10^3 CFU/ml onto five tryptic soy agar (Merck, Darmstadt, Germany) plates. The colonies on the plates were counted after 24 h of incubation at 35 °C. $\bar{x}$ represents the mean value of five plate counts. An aliquot of the 10^4 CFU/ml solution stored from the previous day was diluted $\bar{x}/5$ fold and $\bar{x}/10$ fold to obtain 500 CFU/ml and 1000 CFU/ml, respectively. This procedure resulted in 1 CFU/2 μl and 2 CFU/2 μl, respectively. The cell density in each inoculum was determined by plating 2 μl of the inoculum onto TSA plates 96 times. The colonies were counted after a 24-h incubation at 35 °C.

2.3. Statistical analysis

2.3.1. Characteristics of bacterial counts

To determine whether bacterial cell counts (n = 96) follow a Poisson distribution after adjusting to 1 CFU/2 μl or 2 CFU/2 μl, a likelihood-ratio test of goodness of fit between the experimentally obtained bacterial cell counts (n = 96) and a Poisson distribution estimated by maximum likelihood estimation (MLE) was